Nerve Growth Factor–induced Neurite Outgrowth in PC12 Cells Involves the Coordinate Induction of Microtubule Assembly and Assembly-promoting Factors

DAVID G. DRUBIN,* STUART C. FEINSTEIN,‡ ERIC M. SHOOTER,‡ and MARC W. KIRSCHNER*

*Department of Biochemistry, University of California, San Francisco, San Francisco, California 94143;
‡Department of Neurobiology, Stanford University School of Medicine, Stanford, California 94305

ABSTRACT Nerve growth factor (NGF) regulates the microtubule-dependent extension and maintenance of axons by some peripheral neurons. We show here that one effect of NGF is to promote microtubule assembly during neurite outgrowth in PC12 cells. Though NGF causes an increase in total tubulin levels, the formation of neurites and the assembly of microtubules follow a time course completely distinct from that of the tubulin induction. The increases in microtubule mass and neurite extension closely parallel 10- and 20-fold inductions of tau and MAP1, proteins shown previously to promote microtubule assembly in vitro. When NGF is removed from PC12 cells, neurites disappear, microtubule mass decreases, and both microtubule-associated proteins return to undifferentiated levels. These data suggest that the induction of tau and MAP1 in response to NGF promotes microtubule assembly and that these factors are therefore key regulators of neurite outgrowth.

Extension of neuronal processes is fundamental to establishing the intricate wiring of the nervous system. These processes can bridge vast distances that separate nerve cell bodies from their respective target cells. Electron microscopic analysis of neuronal processes has shown that they are densely packed with parallel arrays of filamentous structures, prominent among which are microtubules (35). Microtubules are present in all eukaryotic cells but especially abundant in neurons where they play an essential role in both the extension and maintenance of neuronal processes (10, 40, 49), and serve also as tracks for vesicular transport between cell bodies and process tips (37, 47). Thus, to understand how these elongated neuronal processes are formed and how vesicular traffic is targeted within them, it is crucial to determine how the spatial and temporal arrangement of microtubule assembly is controlled. In particular, for further biochemical investigations we would first want to know if microtubule assembly in developing neuronal processes involves a rearrangement of pre-existing microtubules or the assembly of new microtubules.

Microtubule assembly has been studied extensively in vitro using neuronal tissue as a source of tubulin and associated proteins. Although pure tubulin monomers assemble poorly into microtubules (tubulin polymers) under physiological conditions, assembly is greatly stimulated by the addition of any of several protein co-factors that bind to microtubules. These co-factors include the tau proteins (8, 48), generally of 50–70 kD, as well as microtubule-associated protein 1 (MAP1)[1] and microtubule-associated protein 2 of ~300 kD (24, 32, 43). Although the action of these co-factors has been studied extensively in vitro, evidence that they promote microtubule assembly in vivo is still lacking. Several studies have shown that the overall activity of microtubule assembly-stimulating factors in brain and neuroblastoma extracts increases markedly during differentiation (30, 39). These findings are consistent with the notion that the extension of neuronal processes by differentiating nerve cells involves net microtubule assembly.

The study of microtubule assembly during neurite extension is facilitated by the use of PC12 cells, a clonal cell line derived from a rat pheochromocytoma (15). PC12 cells project long neurites in response to nerve growth factor (NGF),

[1] *Abbreviations used in this paper:* dbcAMP, dibutyryl cAMP; EB, extraction buffer; LB, lysis buffer; MAP1, microtubule-associated protein 1; NGF, nerve growth factor.

a target-derived protein that is a regulator of the development and maintenance of sympathetic and some sensory neurons (25, 42). PC12 cells are ideally suited for this study since neurite outgrowth is readily induced by NGF and the resulting neurites are filled with microtubules (29).

We have studied in detail the relationship between microtubule assembly and neurite outgrowth in PC12 cells. We find that increases in microtubule levels correlate precisely with increases in neurite length during NGF-induced PC12 cell differentiation. Furthermore, NGF causes total tubulin levels to increase, but the time course for this increase does not correlate with the increase in assembled microtubules, implying that an independent signal is responsible for promoting microtubule assembly. It is particularly significant that levels of two proteins, tau and MAP1, shown previously to promote microtubule assembly in vitro and to co-localize with microtubules in vivo, increase with time courses identical to that of the induction of microtubule assembly and neurite outgrowth. These data suggest that tau and MAP1 serve to promote microtubule assembly in vivo and are therefore key factors that regulate neurite formation during neuronal differentiation.

MATERIALS AND METHODS

Cell Culture: PC12 cells were originally obtained from D. Schubert (Salk Institute, La Jolla, CA). They were grown in Dulbecco's modified Eagle's medium, supplemented with 10% "supplemented" calf serum and 5% horse serum (HyClone Laboratories, Sterile Systems, Inc., Logan, UT). Cells were cultured in a humidified 37°C incubator with a 12% CO_2 atmosphere. Before cells were plated, culture dishes were treated with 100 μg/ml polylysine for 2 h, then rinsed twice with sterile water and once with media without serum. It is important that cells were plated at 1–2 × 10^4 cells/cm^2. The initial plating density can be a major source of variability between experiments. Plating at high density results in excessive cell clumping and severely limits the percentage of cells that extend neurites as well as the density that the neurite network can attain. The extent of the neurite outgrowth response in turn affects the magnitude of the microtubule assembly induction and the microtubule protein level inductions. In addition, initial plating at high density causes tau and tubulin base-line levels to be elevated (see text), further limiting the full observed induction of these proteins.

When appropriate, NGF was added to 100 ng/ml ~12 h after cells were plated. The β-subunit of 7S NGF was purified from mouse salivary glands by the procedure of Smith et al. (44). For some experiments dibutyryl cAMP (dbcAMP) was added to 1 mM. For priming experiments cells were grown in suspension on bacterial petri dishes (Falcon Labware, Oxnard, CA). Average neurite length was determined by measuring 200 neurites for each time point with a Numonics length digitizer (Numonics Corp., Landsdale, PA), in random field photographs of PC12 cells cultured in NGF on three separate plates.

Tau, MAP1, and Tubulin Protein Level Determination: All protein extraction buffers contained 10 μM benzamidine HCl, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml *O*-phenanthroline, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin A to inhibit protein degradation. To prepare PC12 cell extracts, 100-mm culture dishes plated with cells were washed three times with 37°C phosphate-buffered saline (PBS; 0.13 M NaCl, 2 mM KCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.2). Next, 0.5 ml lysis buffer (LB; 25 mM Na_2HPO_4, 0.4 M NaCl, 0.5% SDS, pH 7.2) was applied to the plate. After 3–5 min the viscous lysate was drained into a polypropylene microfuge tube and boiled for 3 min. The lysate was then centrifuged for 10 min in an Eppendorf centrifuge, and DNA-containing pellet was removed.

Protein was determined by the method of Lowry et al. (28). 30 μl extract was assayed in 1-ml reactions. Bovine serum albumin was used to generate standard curves and 30 μl LB was added to each standard reaction to compensate for any interference introduced by constituents of LB in lysate samples.

SDS PAGE was performed on 6 or 8.5% polyacrylamide gels (30:0.8 cross-linking) to resolve MAP1 or tau and tubulin, respectively (27). Separation of α- and β-tubulin was enhanced by raising the pH of the resolving gel to 9.2. Molecular weight standards were transferrin (90,000), bovine serum albumin (68,000), α-tubulin (55,000), β-tubulin (53,000), ovalbumin (45,000), chymotrypsin (25,000), and hemoglobin (15,500).

For tau and tubulin immunoblots, electrophoretic transfer was for 12 h at 50 mA using the conditions described by Burnette (6). Electrophoretic transfer for MAP1 immunoblotting was for 48 h at 30 V in 0.02% SDS, 0.15 M glycine. Microtubule proteins for mass standards were prepared from rat brain by the Weingarten et al. (48) modification of the procedure of Shelanski et al. (41). Rat brain microtubule protein concentration was determined by the procedure of Lowry et al. (28), and relative concentrations of tubulin and MAP1 in the microtubule protein were estimated by densitometry of a Coomassie Blue-stained SDS gel. For tau mass standards, heat stable microtubule protein was prepared from rat brain microtubule protein as described by Herzog and Weber (20). The protein concentration of the heat stable microtubule protein was determined by the procedure of Lowry et al. (28), and the proportion of protein represented by tau was estimated by densitometry of a Coomassie Blue-stained SDS gel.

For quantitative immunoblotting, 30 μg per lane of PC12 cell lysates, and five different amounts of appropriate mass standards (10 ng–2 μg tubulin, 10 ng–1 μg MAP1, 1–200 ng tau), were loaded on SDS gels. Immunoblots were probed with either a MAP1 monoclonal antibody (kindly provided by David Asai) at a 1:500 dilution, DMβ-1 and DMα-1 tubulin monoclonal antibodies (5) at a 1:500 dilution, or affinity-purified tau antiserum (11, 36) at a 1:200 dilution. Iodinated secondary antibodies, used at 10^6 cpm/ml, were prepared from the IgG fraction of goat anti-rabbit or anti-mouse IgG (Cappel Laboratories, Cochranville, PA) by the method of Hunter and Greenwood (21). Autoradiography was on Kodak X-omat AR film, using Dupont Lightening plus intensifying screens. Autoradiographs were aligned with immunoblots, and tubulin, tau, and MAP1 levels were quantitated by scintillation counting of nitrocellulose blot slices in Aquasol (New England Nuclear, Boston, MA). For each set of samples analyzed by quantitative immunoblotting, a duplicate set was subjected to SDS PAGE and the resulting gel was stained with Coomassie Blue to verify that equal amounts of protein were loaded in each lane. Preliminary quantitation of autoradiographic data by densitometry proved inaccurate, presumably due to reciprocity failure (the lack of linearity between radioactive decay and autoradiographic grain density) of the x-ray film. Instead, quantitation by scintillation counting of immunoblot slices gave linear results over wide ranges of protein concentration.

Microtubule Mass Determination: To measure microtubule mass during PC12 cell neurite outgrowth, detergent-extracted cytoskeletons, free of unassembled tubulin, were prepared under microtubule-stabilizing conditions essentially as described by Solomon et al. (46). The tubulin content of the cytoskeletons was measured following the scheme of Caron et al. (Caron, J., manuscript in preparation). In brief, cells were washed once with 37°C PBS and once with extraction buffer (EB; 0.1 M PIPES, 1 mM $MgSO_4$, 2 mM EGTA, 0.1 mM EDTA, 2 M glycerol, pH 6.75). Cells on 100-mm plates were subsequently extracted twice for 8 min with 0.5 ml EB containing 0.1% Triton X-100 and protease inhibitors (listed above). After excess EB was drained from each plate, 0.5 ml LB was added for 3–5 min to solubilize the detergent-extracted cytoskeletons. In addition, the 1 ml EB used to extract PC12 cells was centrifuged for 1 min in an Eppendorf centrifuge to collect insoluble material that came off the culture dish during extraction. This material was added back to the lysis mixture in LB. The viscous cytoskeletal lysate was boiled for 3 min, then centrifuged for 10 min in an Eppendorf centrifuge, and the DNA-containing pellet was removed. The protein concentration of the extracted and cytoskeletal fractions was determined by the Lowry assay. Equal amounts of cytoskeletal protein fraction samples were loaded onto SDS polyacrylamide gels, and the tubulin contents were determined by quantitative immunoblotting, as described above, using rat brain tubulin as mass standards. The protein remaining in the cytoskeleton fraction reproducibly represented 25% of total cellular protein in undifferentiated PC12 cells, consistent with the finding of Solomon et al. (46). Thus, the fraction of each cytoskeleton sample represented by polymeric tubulin was divided by four to determine the percentage of total cellular protein represented by polymeric tubulin. We found that during the course of neurite outgrowth the percentage of total protein not extracted by EB increased. We chose not to compensate for this increase in unextractable material in our calculations of percent total protein in microtubules because the increase did not occur in a regular manner. Thus, the percentage of total protein represented by polymeric tubulin in Fig. 2*a* may be underestimated by 10–25% for the later time points.

RESULTS

Neurite Outgrowth Involves Net Microtubule Assembly

As shown in Fig. 1*a*, PC12 cells cultured in the absence of NGF have a rounded morphology. When these cells are cultured in the presence of NGF for several days, they acquire

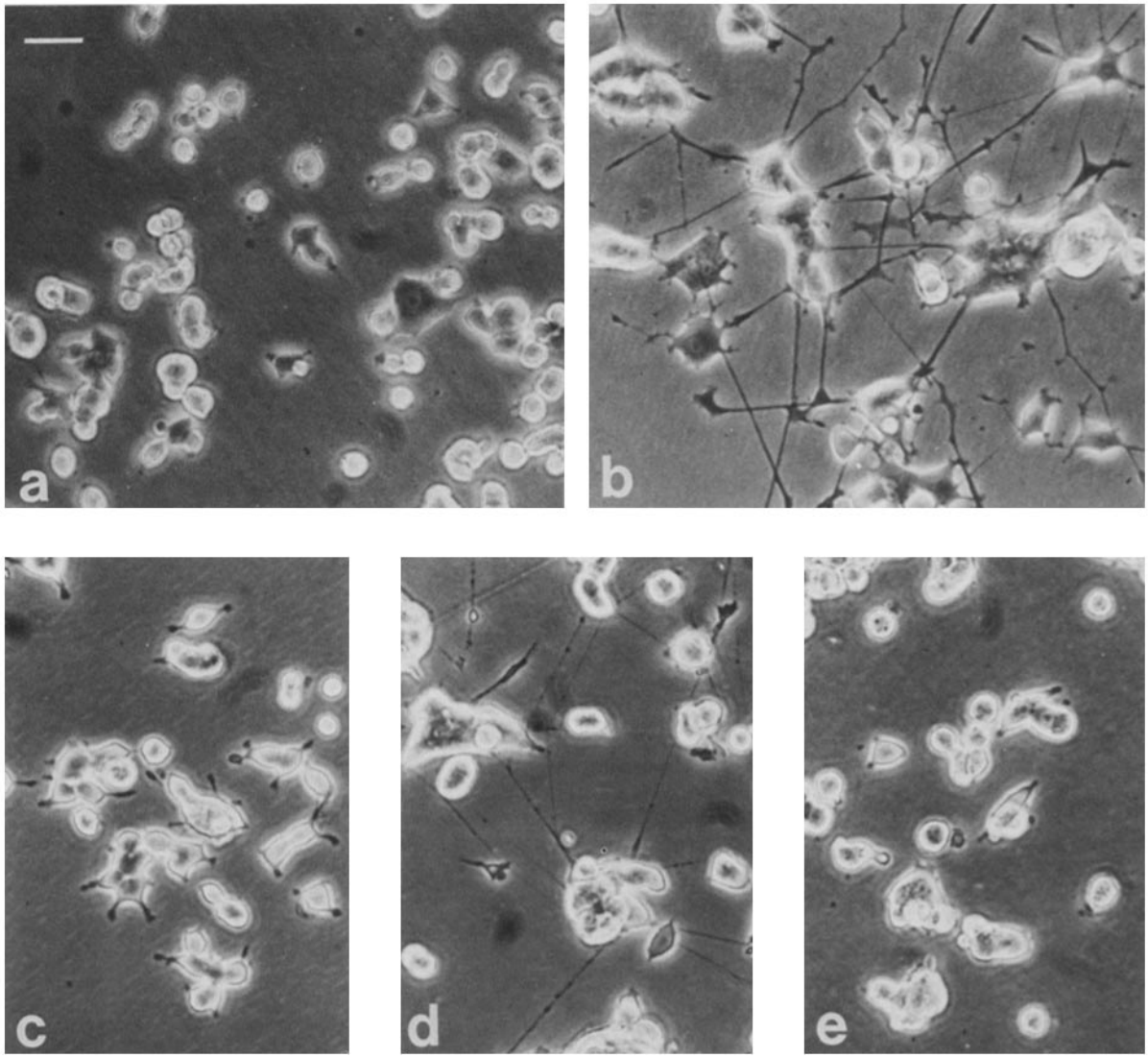

FIGURE 1 PC12 cell neurite outgrowth requires the continued presence of NGF and intact microtubules. (*a*) Undifferentiated PC12 cells. (*b*) PC12 cells cultured in 100 ng/ml NGF for 5 d. (*c*) PC12 cells cultured in 100 ng/ml NGF for 5 d and then withdrawn from NGF for the next 2 d. (*d*) PC12 cells cultured in 100 ng/ml NGF for 7 d and then treated with 10 μg/ml colcemid for 2 h. (*e*) PC12 cells cultured in 100 ng/ml NGF for 7 d and then treated with 10 μg/ml colcemid for 6 h. Bar, 20 μm. × 400.

many characteristics of differentiated neurons (15, 38). Most important for this study, neurites project from the cell bodies (Fig. 1 *b*). Also, maintenance of the neurite network depends upon the continued presence of NGF. NGF-differentiated PC12 cells withdrawn from NGF lose their neurites within 2 d (Fig. 1 *c*). Furthermore, as is the case with true neuronal processes, PC12 cell neurites contain many parallel arrays of microtubules (29). The growth and maintenance of PC12 cell neurites depend on these microtubules since treatment of differentiating (or differentiated) PC12 cells with colcemid for 2–6 h to depolymerize the microtubules causes the neurites to retract or decay (Fig. 1, *d* and *e*). Nocodazole and vinblastine, which also disrupt microtubules, similarly cause neurite loss (data not shown).

To determine if PC12 cell neurite outgrowth involves net microtubule assembly or rearrangement of existing microtubules, the level of polymeric tubulin was measured in PC12 cells cultured for various times in the presence of NGF. Detergent-extracted cytoskeletons, containing only polymeric tubulin, were prepared under microtubule-stabilizing conditions. The tubulin content of these cytoskeletons was measured on immunoblots using defined amounts of purified rat brain tubulin as mass standards. During the time course of NGF administration, average neurite length was also determined. (Since substantial cell clumping occurred in PC12 cell cultures after 7–9 d of growth in NGF, we terminated all experiments in this study after ~1 wk. We concentrated on early events in neurite outgrowth when cells were unclumped and actively extending neurites.) Microtubule mass, expressed as the percentage of total cellular protein, and average neurite length are compared in Fig. 2 *a*. During the first 2–3 d in the presence of NGF little change is observed in microtubule mass and there is no appreciable neurite extension. After 3 d in the presence of NGF, however, microtubule mass and average neurite length increase progressively and in concert. Thus, neurite outgrowth is accompanied by net microtubule assembly, which from day 3 to day 9 of the experiment increases more than twofold. Withdrawal of NGF from PC12

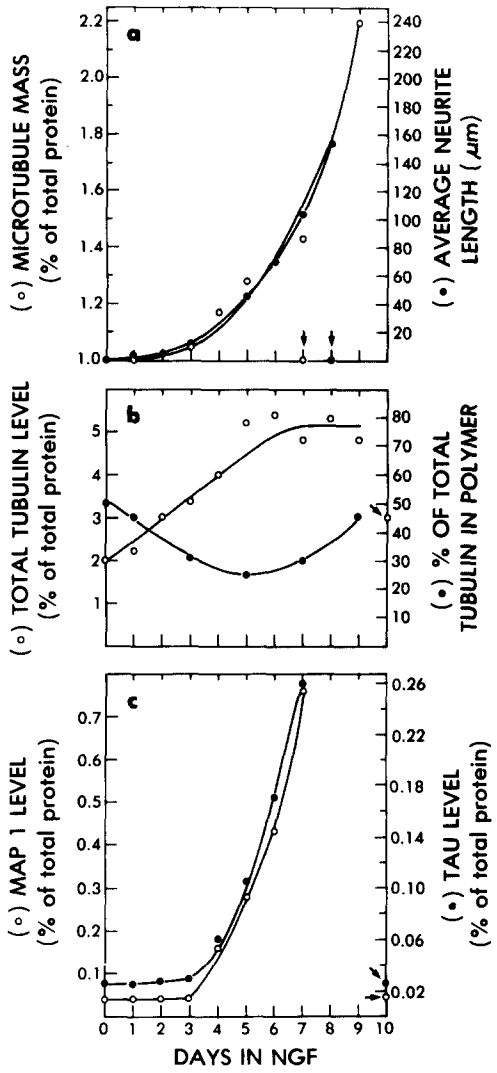

FIGURE 2 Quantitative analysis of neurite length, microtubule mass, and microtubule protein levels during PC12 cell neurite extension. (*a*) Microtubule mass, determined by quantitative immunoblotting of 15 μg detergent-extracted cytoskeleton protein with antitubulin monoclonal antibodies, and average neurite length for 200 neurites measured each day, are plotted as a function of culture time in the presence of NGF. Arrows indicate data points collected after 2 d (○) or 3 d (●) of NGF withdrawal. (*b*) Total tubulin levels, determined by quantitative immunoblotting of 30 μg total PC12 cell protein with antitubulin monoclonal antibodies, are plotted as a function of culture time in NGF. The arrow marks a data point collected after 3 d of NGF withdrawal. The percentage of total tubulin in polymer form was determined from the ratio of microtubule mass in *a* to total tubulin level in *b*. (*c*) MAP1 and tau protein levels, determined by quantitative immunoblotting of 30 μg total PC12 cell protein with a MAP1 monoclonal antibody and an affinity-purified anti-tau serum, are plotted as a function of culture time in the presence of NGF. (Tau quantitation is for 61-, and 68-, and 125-kD polypeptides combined.) Arrows indicate data points collected after 3 d of NGF withdrawal.

cells causes neurites to disappear and microtubule mass to return precisely to the starting levels (Fig. 2*a*, arrows).

Several control experiments demonstrate that the extraction protocol employed cleanly and quantitatively separates assembled from unassembled tubulin. First, treatment of PC12 cells with colchicine for several hours to completely depolymerize microtubles (verified by immunofluorescence) resulted in virtually all tubulin becoming detergent extractable. In addition, taxol treatment to drive microtubule assembly essentially to completion resulted in all tubulin becoming detergent unextractable. Since the microtubule stabilizing conditions used in the extraction procedure are also conditions that favor microtubule assembly, one concern is that the extraction procedure might cause net microtubule assembly during extraction and therefore an overestimation of in vivo tubulin polymer levels. However, Solomon et al. (46) showed that under extraction conditions almost identical to ours, radiolabeled tubulin present in the extraction buffer does not become incorporated into the cytoskeleton, and that the presence of GTP, which is required for assembly, does not affect tubulin retention. Finally, we found that increasing the glycerol concentration in the extraction buffer from 2 to 4 M, which should further favor microtubule assembly, did not increase the proportion of tubulin in the detergent-extracted cytoskeletons.

Tubulin Accumulation Does Not Drive Microtubule Assembly

To test whether an accumulation of tubulin monomers drives microtubule assembly during neurite outgrowth, total tubulin levels in PC12 cells, cultured for various times in the presence of NGF, were analyzed by immunoblotting. As shown in Fig. 3, tubulin levels increase slightly in response to NGF administration over a time course of 7 d (*0–7*), and decrease over 3 d of NGF withdrawal (*−1, −2, −3*). However, as can be seen in Fig. 2*b* (open circles), quantitative analysis of this data clearly demonstrates that the time course of tubulin induction is completely distinct from that of the induction of microtubule assembly. Tubulin levels increase from day zero without any lag phase whereas microtubule levels remain unchanged during the first 3 d of NGF treatment and then begin to increase. Since the tubulin induction precedes the microtubule assembly induction by several days, it is apparent that the percentage of tubulin in the polymer form actually declines intially; it then rises several days later after new microtubule assembly is stimulated (Fig. 2*b*, closed circles). These data suggest that tubulin accumulation does not

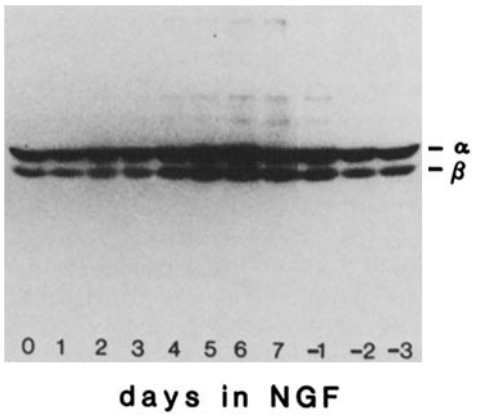

FIGURE 3 Changes in total tubulin levels in response to NGF. Immunoblot of PC12 cell extracts probed with an anti-tubulin antiserum. PC12 cell extracts were prepared from cells cultured in NGF for 0–7 d. After 7 d, NGF was withdrawn from the cells and extracts were prepared after 1–3 d of withdrawal (*−1, −2, −3*). 30 μg of protein was loaded in each lane. Faint bands above tubulin bands are explained in the legend to Fig. 4.

drive microtubule assembly during neurite outgrowth. Rather, they suggest that a signal distinct from the accumulation of tubulin monomers must exist to promote microtubule assembly.

Tau and MAP1 Accumulate in Concert with the Induction of Microtubule Assembly

We next examined the question of whether microtubule assembly during neurite outgrowth might be induced by increasing levels of protein co-factors known to promote microtubule assembly in vitro. In earlier studies, two well-characterized microtubule-associated proteins identified in neuronal tissue, tau and MAP1, were found in PC12 cells (12, 16). A third well-known co-factor, MAP2, was not found. Employing biochemical and immunocytochemical criteria, we have previously shown that both tau and MAP1 are bound to microtubules in PC12 cells (12). In addition, Greene et al. (17) showed that MAP1 levels are elevated in differentiated PC12 cells. We now examined the levels of tau and MAP1 in PC12 cells as a function of time cultured in the presence of NGF. Fig. 4*a* shows an immunoblot probed with an affinity-purified antiserum raised against bovine brain tau protein. (This identical blot was subsequently probed with a tubulin antiserum to generate the autoradiogram shown in Fig. 3. The faint bands seen above the tubulin bands in that figure are residual tau bands.) The three polypeptides recognized by this antiserum, migrating at 61, 68, and 125 kD, are coordinately induced in response to NGF treatment, and all return to undifferentiated levels when NGF is withdrawn. In addition to being recognized by the affinity-purified anti-tau antiserum, these proteins are closely related in primary sequence, as shown by peptide mapping and all bind to microtubules in vitro (Drubin, D. G., and M. W. Kirschner, manuscript in preparation). Fig. 4*b* shows an immunoblot probed with a monoclonal MAP1 antibody. As with the tau proteins, MAP1 is induced in response to NGF treatment, and deinduced when NGF is withdrawn.

Tau and MAP1 data from several experiments are presented quantitatively in Fig. 2*c* (Tau quantitation is for the 61-, 68-, and 125-kD polypeptides together). During the first 3 d in the presence of NGF, when little change in neurite length or microtubule mass is occurring (Fig. 2*a*), tau and MAP1 levels remain unchanged at 0.025 and 0.04%, respectively, of the total cellular protein. After 3 d of exposure to NGF tau and MAP1 levels increase in concert with microtubule assembly and neurite outgrowth. By day 7, tau and MAP1 levels have increased to 10- and 20-fold above starting levels and now represent 0.25 and 0.8% of the total protein, respectively. As discussed earlier, withdrawal of NGF from PC12 cells causes neurites to disappear and microtubule levels to decline within 2 d to the undifferentiated levels. Similarly, NGF withdrawal causes tau and MAP1 levels to return rapidly to their levels in undifferentiated cells (Fig. 2*c*, arrow), with MAP1 levels decreasing slightly faster (compare Fig. 4*a* and *b*). Thus, unlike tubulin levels, tau and MAP1 levels correlate closely with microtubule polymer levels both in the assembly and disassembly phases.

Microtubule Protein Accumulation in Response to Stimuli Other than NGF

Several stimuli other than NGF can induce neurites or can, in conjuction with NGF, affect the time course of neurite extension in PC12 cells. Having shown that neurite outgrowth involves net microtubule assembly, we sought to determine if factors that alter the rate or pattern of PC12 cell neurite outgrowth also affect tau, MAP1, and tubulin levels in a manner consistent with a model in which induced tau and MAP1 levels mediate net microtubule assembly. The first situation examined was growth of PC12 cells in dbcAMP, an agent that induces PC12 neurites slightly faster than does NGF (18). As seen in Fig. 5*a*, dbcAMP produces a more rapid induction of tau protein than does NGF. dbcAMP also induces MAP1 more rapidly than does NGF, although in this case to a lesser extent than NGF (compare Fig. 5*b* with Fig. 4*b*). A second case studied by Gunning et al. (18) is the administration of both NGF and dbcAMP together. These agents act synergistically on PC12 neurite growth, causing neurites to grow much more quickly than with either agent alone. Treatment of PC12 cells with NGF plus dbcAMP causes both tau and MAP1 to be quickly and strongly induced (Fig. 5, *a* and *b*). Thus, stimuli that increase the rate of neurite outgrowth increase the rate of tau and MAP1 induction, consistent with the conclusion that these factors promote microtubule assembly during neurite outgrowth.

Neurite outgrowth in response to NGF can be prevented

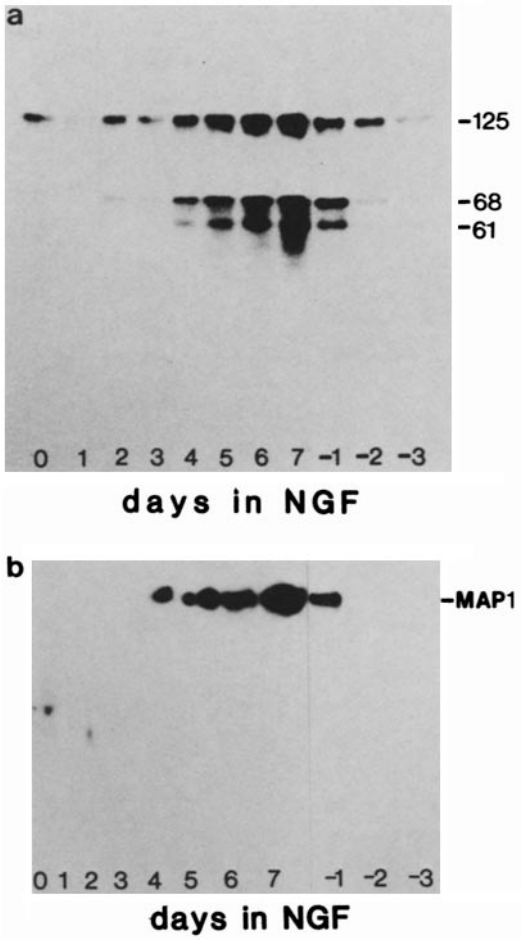

FIGURE 4 Changes in tau and MAP1 levels in response to NGF. (*a*) The same blot shown in Fig. 3 was probed here with an affinity-purified anti-tau antiserum. The faint bands seen above the tubulin bands in Fig. 3 represent residual tau staining since the anti-tubulin antiserum was applied to the blot subsequent to the anti-tau antiserum. (*b*) Immunoblot probed with monoclonal MAP1 antiserum.

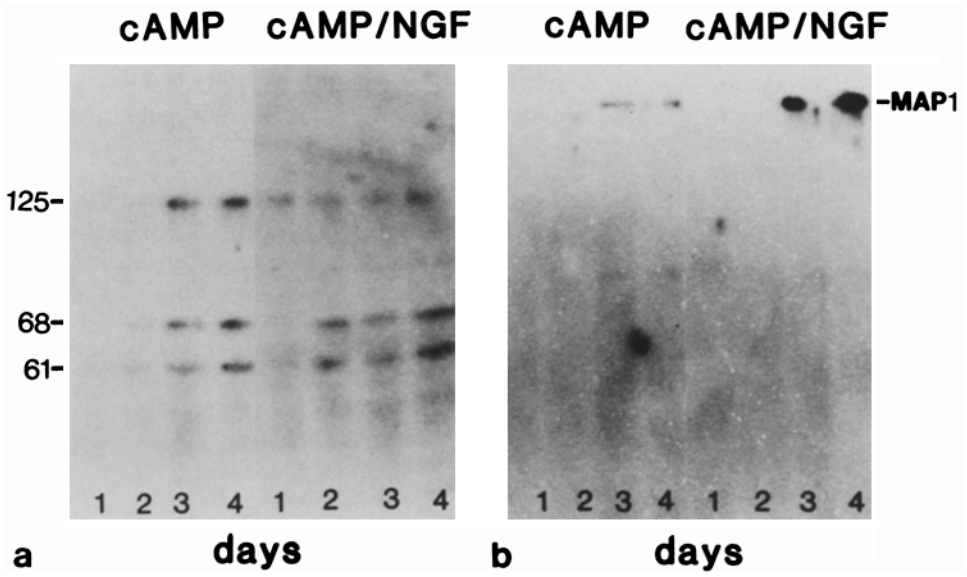

FIGURE 5 Tau and MAP1 induction by dbcAMP or NGF plus dbcAMP. Immunoblots of PC12 cell extracts probed with tau antiserum (*a*) or MAP1 monoclonal antiserum (*b*). PC12 cells were cultured in the presence of dbcAMP or NGF plus dbcAMP for 0–4 d.

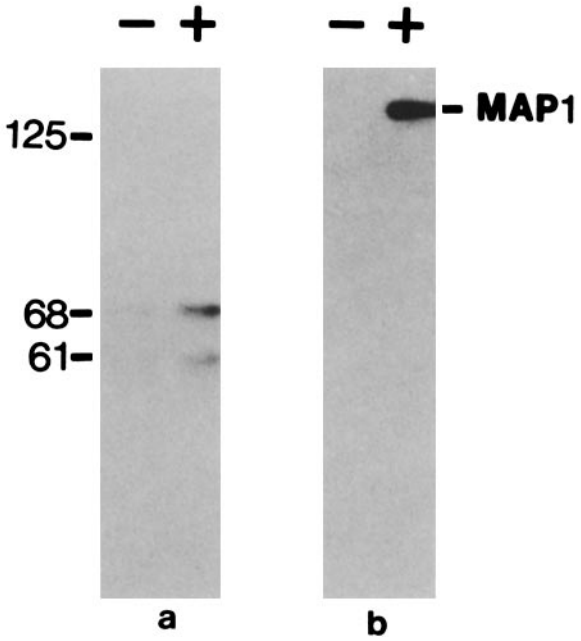

FIGURE 6 Elevation of tau and MAP1 in "primed" PC12 cells. Immunoblots of PC12 cell extracts probed with tau antiserum (*a*) or MAP1 monoclonal antiserum (*b*). PC12 cells were cultured on bacterial petri dishes for 4 d in the absence (–) or presence (+) of 50 ng/ml NGF. PC12 cells do not extend neurites on petri dishes.

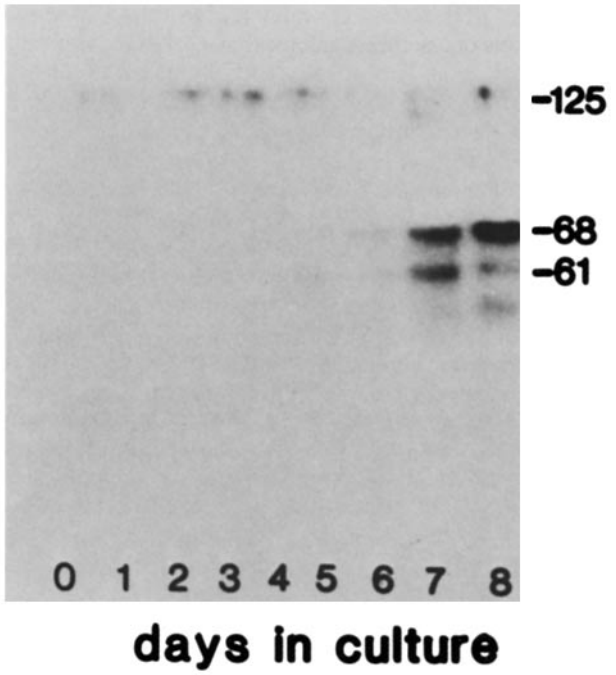

FIGURE 7 Induction of tau in response to cell crowding. Immunoblot of PC12 cell extracts probed with tau antiserum. Approximately 10^6 PC12 cells were plated onto each 100-mm plate. Cells were cultured for 0–8 d in the absence of NGF. Under these conditions PC12 cells continue to proliferate and do not extend neurites.

by denying PC12 cells access to an adhesive substrate. However, if PC12 cells are first grown in suspension in the presence of NGF and are then plated on an adhesive substrate, still in the presence of NGF, they grow neurites without the lag phase shown in Fig. 2*a* (16). This "priming" has been interpreted to result from the accumulation of limiting factors required to extend a neurite. Presumably, some of these factors may be those necessary to build new microtubules. Consistent with this idea, Greene et al. (17) found that MAP1 levels are elevated in primed PC12 cells. We concur with this result (Fig. 6*b*) and also find that tau levels are elevated in primed PC12 cells (Fig. 6*a*). Again, these results are consistent with the conclusion that tau and MAP1 support microtubule assembly in PC12 cells.

Finally, it has been found that crowding of PC12 cells can duplicate some of the biochemical changes caused by NGF, such as increasing choline acetyltransferase specific activity, but does not cause neurite outgrowth (14). We found that cell crowding causes a tau protein induction (Fig. 7) but no induction of MAP1 (not shown).

DISCUSSION

Neurite Outgrowth Involves Net Microtubule Assembly

The mechanisms by which NGF regulates axon growth or

regrowth and maintains the differentiated phenotype are not yet understood. For some time, however, it has been known that neuronal process extension and maintenance require intact microtubules. In fact, microtubules may be solely responsible for providing the force required to extend and maintain neuronal processes (31), whereas actin filaments may provide an opposing force in the neurite (45). One effect of NGF, then, would be to promote microtubule assembly in a growing process and to maintain those microtubules in a differentiated process. Therefore, the first question we would want to address is, Does the formation of microtubules in a growing neuronal process involve a rearrangement of existing microtubules or the assembly of new ones?

We found that microtubule mass increases precisely in parallel with neurite extension (Fig. 2*a*). This result is consistent with an earlier finding that differentiated neuroblastoma cells contain higher levels of assembled tubulin than do undifferentiated cells (34). When NGF is withdrawn from differentiated PC12 cells, microtubule levels return to those seen in undifferentiated cells as neurites disappear. Thus, one effect of NGF is to stimulate net microtubule assembly as neurites are extended, and another is to maintain the increased levels of assembled microtubules.

Tubulin Accumulation Does Not Drive Microtubule Assembly

One possible mechanism to drive microtubule assembly during neurite outgrowth involves raising total tubulin levels in PC12 cells above the steady state level, thus driving tubulin polymerization. Net assembly should occur until the tubulin monomer concentration again reaches the steady state level. In this model, tubulin subunits are limiting for assembly.

We found that tubulin levels do indeed increase 2.5-fold in response to NGF (Fig. 2*b*, open circles). However, the tubulin induction precedes the microtubule assembly induction by ~3 d (Fig. 2*a*, open circles). Tubulin thus does not appear to be limiting for net assembly. Furthermore, the data in Fig. 2*b* (closed circles) indicate that, even when net microtubule levels first begin to increase, the percentage of total tubulin in the polymeric form is still lower than in the undifferentiated state. This is because at first total tubulin levels increase faster than polymeric tubulin levels. Thus, tubulin is not limiting for assembly even after net assembly has been induced.

Several other investigators have also reached the conclusion that tubulin levels may not be limiting for microtubule assembly during neuronal process extension. First, tubulin levels in differentiating brain tissue or neuroblastoma cells do not change appreciably at the time of neurite extension (13, 33, 34, 40), even though process formation seems to involve net microtubule assembly (13, 34). Second, Heidemann et al. (19) investigated microtubule stability to anti-microtubule drugs in PC12 cells differentiated under conditions that increase the rate of neurite growth. They found that microtubules were most stable under conditions that produced the fastest neurite outgrowth. They speculated that the increased stability of fast-growing neurite microtubules might result from increased levels of factors that favor microtubule assembly and usually limit the rate of neurite extension. The conclusion that tubulin levels are not limiting for microtubule assembly has also been reached by investigators studying non-neuronal systems. As frog oocytes develop into unfertilized eggs and then become activated, a progressive and dramatic increase in the potential of the cytoplasm to support microtubule assembly occurs while the tubulin concentration remains unchanged (23). It appears that these various cells have evolved strategies to regulate microtubule assembly that do not require drastic changes in total tubulin levels each time environmental signals dictate that changes in levels of assembled tubulin are needed.

The question therefore remains, What regulates microtubule assembly during neurite outgrowth? One possibility is that physiological conditions, such as GTP or calcium ion concentrations, could limit assembly. Alternatively, negative protein factors could inhibit assembly, or positive co-factors favoring assembly might be limiting. Changes in the activity of negative or positive regulators of assembly could be manifested by changes in their levels, or by changes in their form by posttranslational modifications. Whatever the nature of the change, the data presented here strongly predicts that it will occur in PC12 cells after 3 d of growth in the presence NGF, when microtubule levels begin to rise markedly.

Microtubule Assembly-promoting Factors Are Induced in Parallel with the Induction of Microtubule Assembly

The well-characterized microtubule-associated proteins identified in brain are good candidates for the limiting factors that drive microtubule assembly during neurite outgrowth. They promote the otherwise poor assembly of tubulin in vitro, but evidence that they have the same function in vivo is lacking. Consistent with the possibility that these factors promote microtubule assembly during neuronal process extension, extracts from differentiated neuroblastoma cells or developed brain tissue have significantly higher levels of microtubule-polymerization promoting activity than do the corresponding less-differentiated sources (30, 39). Also, many groups have reported that the microtubule-associated protein composition changes during brain development (3, 11, 30). We took advantage of the relatively uniform response of PC12 cells to NGF to analyze microtubule-associated protein levels during neurite outgrowth and microtubule level induction.

MAP1 has been identified in PC12 and N115 neuroblastoma cells and found to be present in higher levels after neurite outgrowth (13, 17). Also, we have previously identified and characterized tau protein in PC12 cells (12). In the present study we found that tau protein and MAP1 increase 10- and 20-fold, respectively, in response to 7 d of growth in the presence of NGF. The time course of neurite outgrowth, the induction of microtubule assembly, and increases in tau and MAP1 levels all occur exactly in parallel and show the same 3-d lag (Fig. 2, *a* and *c*). The induction of tubulin, on the other hand, occurs with no lag phase and does not correspond to the induction of microtubule assembly (Fig. 2, *a* and *b*). When NGF is withdrawn from differentiated PC12 cells, tau and MAP1 levels decline as microtubule polymer levels decline and neurites disappear. These results strongly suggest that tau and MAP1 are limiting factors for microtubule assembly during neurite outgrowth. To test this assertion directly we are currently trying to alter tau and MAP1 levels in differentiating PC12 cells by microinjecting the cells with purified tau and MAP1 or antibodies raised against them.

Despite the 2.5-fold increase in tubulin level, the ratios of tau and MAP1 to total tubulin, and to assembled tubulin, increase dramatically during neurite outgrowth. Using

TABLE I. *Approximate Molar Ratios of Tau and MAP1 to Total and Assembled Tubulin*

	Undifferentiated	Differentiated
Tau/total tubulin	1:68	1:17
MAP1/total tubulin	1:175	1:25
Tau/assembled tubulin	1:34	1:5
MAP1/assembled tubulin	1:88	1:7

100,000, 350,000, and 85,000 as the respective molecular weights of the tubulin dimer, MAP1, and PC12 tau (average of 125, 68, and 61 kD), and the percentage of total protein represented by each protein (Fig. 2), we have calculated the approximate molar ratios of tau and MAP1 to total and assembled tubulin before NGF treatment and after full induction (Table I). During neurite outgrowth ratios of tau to total tubulin increase from 1:68 to 1:17, and ratios of MAP1 to total tubulin increase from 1:175 to 1:25. These changes in tau and MAP1 stoichiometries with respect to tubulin are well within the effective ranges for promoting assembly determined in in vitro studies using physiological tubulin concentrations (7, 20, 26). We conclude that the inductions of tau and MAP1 we observe are well within the effective ranges for promoting microtubule assembly on the scale observed during neurite outgrowth.

Since we have previously shown that essentially all of the tau and MAP1 in PC12 cells is bound to microtubules (12), we can use the data in Fig. 2, *a* and *c* to estimate the molar ratios of these co-factors to assembled tubulin. Growth in NGF increases approximate tau-to-assembled tubulin ratios from 1:34 to 1:5, whereas approximate MAP1 ratios increase from 1:88 to 1:7. These estimated ratios are close to saturating ratios determined in in vitro studies (1, 7). Since microtubules in differentiated PC12 cells are coated with such high levels of tau and MAP1, they should be markedly more stable than microtubules in undifferentiated PC12 cells. This in fact has been observed (4, 19).

Microtubule Protein Accumulation in Response to Stimuli Other than NGF

By examining the effects of culturing PC12 cells under conditions that alter the neurite outgrowth response, we have obtained additional evidence that tau and MAP1 are limiting factors during neurite extension. When the rate of neurite outgrowth is increased in response to either dbcAMP or dbcAMP plus NGF, tau and MAP1 are induced more rapidly. Furthermore, both tau and MAP1 are induced in primed PC12 cells, and those cells show no lag phase when plated on an adhesive substrate (results summarized in Table II). Note that primed cells fail to project neurites when plated in the presence of NGF and cycloheximide (Feinstein, S. C., manuscript in preparation), which suggests that synthesis of new proteins in addition to tau and MAP1 is necessary for neurite outgrowth. Therefore, though we feel that tau and MAP1 are necessary for neurite outgrowth, they may not be sufficient to produce neurite outgrowth.

It is important to note that the induction of tau and MAP1 can be uncoupled. First, dbcAMP induces tau to a much greater extent than it induces MAP1. Neurites that grow in response to dbcAMP alone are shorter and less stable than those produced by NGF (18). Also, tau induction precedes

TABLE II. *Microtubule Protein Accumulation in Response to Various Stimuli*

	Neurite growth	Tubulin induction	Tau induction	MAP1 induction
NGF	Normal	+	+++	+++
dbcAMP	Fast	+	+++ (fast)	+ (fast)
NGF + dbcAMP	Very fast	+	+++ (fast)	+++ (fast)
Suspension (+NGF)	–	+	++	+++
Crowding (–NGF)	–	+ (slow)	+ (slow)	–

–, no response. +, slight induction. ++, moderate induction. +++, large induction.

the MAP1 induction in cells treated simultaneously with NGF and dbcAMP. These data suggest that tau may be sufficient to promote the early phase of neurite outgrowth but that MAP1 is essential for promoting microtubule stability and neurite elongation. In addition, cell crowding induces tau but not MAP1. The modulation of tau in this situation is interesting with respect to the work of Karsenti et al. (22), who found that the cytoplasm of crowded mouse L cells more readily supports microtubule assembly than does that of uncrowded L cells. Perhaps the induction of tau in crowded PC12 cells is a manifestation of a general tendency of cells to favor higher levels of microtubule assembly when faced with crowding.

Finally, all of the stimuli tested cause a modest induction in PC12 tubulin levels (Table II). The tubulin induction could result from the well-characterized autoregulatory control over tubulin synthesis (2, 9). Perhaps when net microtubule assembly occurs during neurite outgrowth, free tubulin levels diminish and, as a result, more tubulin is made. We have shown that this regulatory pathway can function in PC12 cells in response to colchicine (unpublished observation). However, while this mechanism may operate after 3 d of growth in the presence of NGF, it clearly is not the dominant mechanism before then. As shown in Fig. 2, *a* and *b*, tubulin levels increase during the first 3 d of growth in the presence of NGF, before the increase in microtubule assembly. Thus, we can say that a mechanism independent of the tubulin autoregulatory mechanism operates and in fact overrides the autoregulatory mechanism to increase total tubulin levels (despite elevated tubulin monomer levels) in anticipation of a need for more tubulin subunits to assemble microtubules in growing neurites. The coordinate synthesis and assembly of the major components of the microtubule cytoskeleton is essential for the morphogenesis of neuronal processes. Understanding how the synthesis of these components is regulated will be an important part of understanding how a neurite is assembled in response to NGF.

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